J Periodont Res 2008; 43: 450–458

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01088.x

Inhibition of the formation of oral calcium phosphate precipitates: the possible effects of certain honeybee products

S. Hidaka[1], Y. Okamoto[2], K. Ishiyama[3], K. Hashimoto[3]

[1]Department of Dental Hygiene, Fukuoka College of Health Sciences, Tamura, Sawara-Ku, Fukuoka, Japan, [2]Department of Dental Engineering, Bioengineering Section, Fukuoka Dental College, Tamura, Sawara-Ku, Japan and [3]Institute for Bee Products & Health Science, Yamada Apiculture Center Inc., Kagamino-Cho, Tomata-Gun, Okayama, Japan

Hidaka S, Okamoto Y, Ishiyama K, Hashimoto K. Inhibition of the formation of oral calcium phosphate precipitates: the possible effects of certain honeybee products. J Periodont Res 2008; 43: 450–458. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard

Background and Objective: We studied the effects of honeybee products on the *in vitro* formation of calcium phosphate precipitates.

Material and Methods: Screening tests of the *in vitro* formation of calcium phosphate precipitates using 20 types of honey and four types of propolis were carried out using the pH drop method.

Results: The inhibitory effect on the rate of amorphous calcium phosphate transformation to hydroxyapatite and on the induction time varied greatly among the 20 types of honey and four types of propolis. We classified them according to their effects on decreasing the rate of amorphous calcium phosphate transformation to hydroxyapatite and/or increasing the induction time. Two of the 20 honeys showed little or no inhibition, either on the rate of amorphous calcium phosphate transformation to hydroxyapatite or on the induction time. Six of the honeys reduced the rate of amorphous calcium phosphate transformation to hydroxyapatite by 12–35% and with a 2.5- to 4.4-fold increase in the induction time. The remaining 12 honeys showed even greater activity. Because four of these 12 honeys had an inhibitory effect on the rate of amorphous calcium phosphate formation, they were excluded as candidates for anticalculus agents. Furthermore, three of the four types of propolis showed an inhibitory effect that was the same as or greater than 1-hydroxyethylidene-1,1-bisphosphonate.

Conclusion: These results suggest that eight honeys and three types of propolis may have potential as anticalculus agents in toothpastes and mouthwashes.

Saburo Hidaka, Department of Dental Hygiene, Fukuoka College of Health Sciences, 2-15-1, Tamura, Sawara-Ku, Fukuoka 814-0193, Japan
Tel: +81 92 801 0411, ext. 108
Fax: +81 92 801 4473
e-mail: sabrnrn@college.fdcnet.ac.jp

Key words: honey; propolis; screening; calcium phosphate precipitation; oral calcification

Accepted for publication January 25, 2008

Honey is a nectar and propolis is a resinous sealant used in the hive; both are commonly collected by honeybees from a wide variety of plants. Honey and propolis have a long history of being used in folk medicine and are now being rediscovered in modern medicine owing to harboring certain pharmacological effects, such as antimicrobial, antioxidant, anti-inflammatory and anti-tumour activities (1,2). Furthermore, both honey and propolis are very useful honeybee products for use in oral health. For example, honey has been reported to be effective in the treatment of periodontal diseases, mouth ulcers and other problems of oral health (3), whereas propolis has been reported to be protective against dental caries because of its antimicrobial activity against a large array of oral microorganisms (4).

However, no work has been reported on the relationship between

honeybee products and oral calcification, which includes both remineralization of tooth enamel and dental calculus formation. The remineralization of tooth enamel can be facilitated by endogenous and exogenous factors such as salivary components, antibacterials, fluoride from extrinsic sources and dietary sources (5). The level of calculus formation is affected by several factors, including age, gender, diet, oral hygiene and diabetes (6). It has been suggested that dental calculus can contribute to the progression of gingivitis or periodontal diseases because its porous structure can retain potentially toxic substances, including stimulators of bone resorption (6,7). In recent years, there has been considerable interest in agents useful for the prevention of calculus formation (8).

Editempa and sodium etidronate have been shown to be effective in decreasing dental calculus in humans and dogs (9,10). More recently, dentifrices containing zinc chloride, pyrophosphate and polypyrophosphate have been developed as anticalculus agents (8,11,12). These chemically synthesized agents are effective inhibitors of hydroxyapatite growth. However, it has been claimed that synthetic chemicals have side effects, whereas natural products have been shown to have comparatively few, or even no, side effects. We have previously screened a number of traditional Chinese (Kampo) medicines *in vitro* and found that nine of them displayed efficacy as anticalculus agents (13). Therefore, we undertook an *in vitro* screening of honeybee products to help determine their influence on *in vivo* oral calcification.

We used the pH drop method to follow the reaction *in vitro* (14). Figure 1 shows an example of the pH record. When phosphate and calcium ions are mixed at pH 7.4, the formation of amorphous calcium phosphate is determined as the first pH drop and its transformation to hydroxyapatite *in vitro* can be followed by the second pH drop (14). The changes occur in two distinct steps. The effectiveness of any agents that affect the calcium phosphate precipitation can be experimentally studied by measuring (i) the rate of pH decrease in the first step, (ii) the onset time of the second rapid decrease and (iii) the rate of pH drop in the second step (14). Some compounds and ions are stimulatory (15), while others are inhibitory or not effective (14,16). As this pH drop method is suitable for obtaining many data points from a limited supply of compound, we have used it for screening many Chinese traditional (Kampo) medicines and metal ions for dental alloys (13,16).

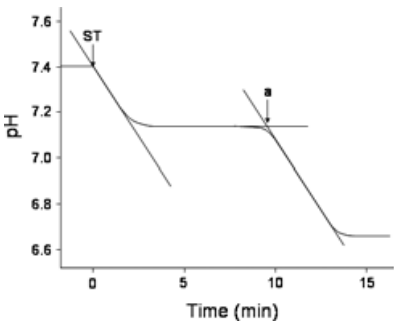

Fig. 1. Representative recording of the rate of pH change induced by the addition of phosphate (ST) to the reaction mixture. Calcium (3 mM) and phosphate (3 mM) were used. The final volume of the assay solution, containing 2 mM Hepes buffer (pH 7.4), was 2 mL. The reaction mixture was stirred at 37°C. Changes occured in two distinct steps. The rate of ΔpH/min was determined by drawing a tangent of the first slope (for the rate of amorphous calcium phosphate formation) and the second slope (for the rate of transformation of amorphous calcium phosphate to hydroxyapatite), respectively. Induction time was determined between ST and the point 'a', which is the intersection 'a' between the baseline and the extended line of the tangent of the second slope.

We carried out the screening test on 20 types of honey and four types of propolis to determine the *in vitro* formation of calcium phosphate precipitates.

Material and methods

Honeybee products and chemicals

The merchandise name and the relevant information of 20 types of commercially available honey from 10 different countries are shown in Table 1. The water extracts of four types of propolis and the relevant information are shown in Table 2. All apirary products were gifts of the Yamada Apiculture Center Inc. (Okayama, Japan). A 60% solution of 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) was purchased from Tokyo Kasei (Tokyo, Japan). Glucose, fructose, sucrose and maltose were purchased from Sigma Aldrich Japan Co. Ltd. (Tokyo, Japan). All other reagents were purchased from ABIOZ Co. Ltd. (Osaka, Japan).

Measurements of pH

A pH meter (F-21; Horiba, Tokyo, Japan) with a combination-type pH electrode (6378-10D; Horiba, Tokyo, Japan) and a recorder were used for pH measurements. The final volume of assay solution was 2 mL (14). The reaction mixture was stirred continuously during the pH measurement, and the temperature was maintained at 37 ± 0.1°C.

Assay of amorphous calcium phosphate formation, its transformation to hydroxyapatite and the induction time, using the pH drop method

Both stock solutions – 100 mM $Ca(NO_3)_2$ and 100 mM KH_2PO_4 – were prepared in 2 mM Hepes buffer (pH 7.4). To 1.88 mL of buffer solution (2 mM Hepes buffer, pH 7.4), 60 μL of the calcium stock solution was added. Then, 60 μL of the phosphate stock solution was added to start the reaction. The final concentrations of calcium and phosphate were both 3 mM.

For the screening study of the honeys, each of the following types of honey, diluted 1:2, was added to the reaction buffer at a final concentration of 1.25–5.0% (v/v), 5 min before the addition of phosphate: Akashia (Romania), Kanro (Romania), Manuka (New Zealand), Orenji (Mexico), Kuroba (Canada), Petasonkasu (Australia), Yukari (Australia), Kohi (Vietnam), Renge (China), Akashia-C (China), Razuberi (USA), Benibana (USA), Burakkuberi (USA), Pepaminto (USA), Seji (USA), Rozumari (Spain), Satoyama-no-soba (Japan), Satoyama-no-soyogo (Japan), Satoyama-no-renge (Japan) and Satoyama-no-hyakka (Japan). For the

Table 1. Merchandise name, plant sources, scientific name and countries of the types of honey used in this study

Merchandise name of honeys	Plant sources	Scientific name	Countries
(Europe)			
Akashia	False acacia	*Robinia pseudoacacia* L.	Romania
Kanro	Honeydew	–	Romania
Rozumari	Rosemary	*Rosmarinus officinalis* L.	Spain
(Oceania)			
Manuka	Manuka	*Leptosepermum-scoparium*	New Zealand
Petasonkasu	Patterson's Curse	*Echium plantagineum*	Australia
Yukari	Eucalyptus	*Eucalyputus globulus*	Australia
(North America)			
Kuroba	Clover	*Trifolium repens*	Canada
Razuberi	Rasepberry	*Rubus idaeus var. strigosus*	USA
Benibana	Safflower	*Carthamus tinctorius* L.	USA
Burakkuberi	Blackberry	*Rubus fructicosus Agg.*	USA
Pepaminto	Peppermint	*Mentha piperita*	USA
Seji	Sage	*Salvia officinalis*	USA
Orenji	Orenge	*Citrus sinensis*	Mexico
(Asia)			
Kohi	Coffee	*Caffea canephora Peirr.ex Froeh.*	Vietnam
Renge	Astragalus	*Astragalus sinicus* L.	China
Akashia-C	False acacia	*Robinia pseudoacacia* L.	China
Satoyama-no-soba	Buckwheat	*Fagopyrum esculentum*	Japan
Satoyama-no-soyogo	Astragalus	*Ilex pedunculosa*	Japan
Satoyama-no-renge	Milk vetch	*Astraglus sinicus* L.	Japan
Satoyama-no-hyakka	Mixed	–	Japan

Some of these names are Japanese versions of what were originally English names.

Table 2. Plant source, scientific name and country in water extracts from propolis used in this study

Propolis	Plant sources	Scientific name	Country
Green propolis-1	Alecrim do Camp	*Baccharis dracunculiforia* DC.	Brazil
Green propolis-2	Alecrim do Camp	*Baccharis dracunculiforia* DC.	Brazil
Green propolis-3	Alecrim do Camp	*Baccharis dracunculiforia* DC.	Brazil
Brown propolis	Unknown	Unknown	Brazil

screening of propolis, each of the following water extracts of propolis were added to the reaction buffer at a final concentration of 250–1000 μg/mL, 5 min before the addition of phosphate: Green propolis-1, Green propolis-2, Green propolis-3 and Brown propolis. Ten to 60 μM of HEBP was also used for comparison. In order to investigate the effect of glucose [2.0% (w/v) = 0.11 M], fructose [2.0% (w/v) = 0.11 M], sucrose [1.0% (w/v) = 0.03 M] and maltose [1.0% (w/v) = 0.03 M], each of these was added separately to the reaction chamber in the absence of honey.

The formation of amorphous calcium phosphate (p.p.m. of Ca^{2+}/min), its transformation to hydroxyapatite (HAP; p.p.m. of Ca^{2+}/min) and the induction time (min) were measured by recording the pH decrease. The rate of pH decrease was converted to the rate of consumption of calcium (P.P.M/10^6/min) (14). The induction time was determined according to the method of Blumenthal *et al.* (17).

Statistics

Data were obtained from three to five measurements and are expressed as the means ± standard deviations. Statistical comparisons were made by analysis of variance and Scheffe's test using a statistical software program. The difference was considered significant when the p value was < 0.05.

Results

Effects of honey on calcium phosphate precipitation

As shown in Table 3, at a concentration range of 2.5–5.0% (v/v), 10 types of honey (Akashia, Kanro, Rozumari, Manuka, Kuroba, Burakkuberi, Pepaminto, Orenji, Akashia-C and Satoyama-no-soyogo) had an inhibitory effect on the rate of formation of amorphous calcium phosphate. The remaining 10 types of honey had no inhibitory effect on the rate of formation of amorphous calcium phosphate. At a concentration range of 1.25–2.5% (v/v), all types of honey, except for Akashia and Satoyama-no-renge, had a significant inhibitory effect on the rate of transformation of amorphous calcium phosphate to hydroxyapatite. When expressed as the percentage of the control rate at a concentration of 2.5% (v/v), the inhibition of the rate of amorphous calcium phosphate transformation to hydroxyapatite ranged from 12 to 88%. All types of honey showed significant inhibitory effects on the increase of the induction time. The increase in ratio ranged from 2.2 to 22 times that of the control.

Effects of propolis on calcium phosphate precipitation

As shown in Table 4, four types of propolis had no effect on the rate of amorphous calcium phosphate formation. However, they did have a significant inhibitory effect on both the rate of amorphous calcium phosphate transformation to hydroxyapatite and the induction time. At a propolis concentration of 500 μg/mL, the inhibition of amorphous calcium phosphate transformation to hydroxyapatite ranged from 30 to 74%. The increased ratio of propolis resulted in an increase of the induction time, which became 2.2 to 5.3 times higher than that of the control.

Effects of HEBP on calcium phosphate precipitation

HEBP had no inhibitory effects on the rate of amorphous calcium phosphate formation but did exhibit significant

Table 3. Effect of honey on the formation of amorphous calcium phosphate (ACP), the transformation of ACP to hydroxyapatite (HAP) and the induction time

Honey	Concentration used % (v/v)	Ca^{2+} consumption (p.p.m./min)		Induction time (min)
		ACP	HAP	
None	0	123 ± 12	13.0 ± 1.3	14.8 ± 1.4
Akashia	1.25	123 ± 12	13.7 ± 1.3	21.9 ± 1.9*
	2.5	110 ± 10	13.3 ± 1.3	35.2 ± 3.0*
	5.0	74.7 ± 6.5*	11.4 ± 1.1*	53.0 ± 4.8*
Kanro	1.25	123 ± 11	6.76 ± 0.57*	49.2 ± 4.3*
	2.5	80.2 ± 7.5*	4.16 ± 0.35*	135 ± 12*
	5.0	73.2 ± 6.9*	2.08 ± 0.18*	242 ± 20*
Rozumari	1.25	110 ± 10	7.56 ± 0.62*	35.9 ± 3.0*
	2.5	95.6 ± 9.0*	6.72 ± 0.59*	40.8 ± 3.5*
	5.0	73.8 ± 7.0*	6.00 ± 0.55*	68.5 ± 6.4*
Manuka	1.25	123 ± 12	13.7 ± 1.2	37.1 ± 3.1*
	2.5	114 ± 11	11.4 ± 1.1*	72.1 ± 6.7
	5.0	79.8 ± 6.5*	4.30 ± 0.40*	103 ± 9.8*
Petasonkasu	1.25	123 ± 12	12.5 ± 1.0	31.6 ± 2.7*
	2.5	120 ± 11	5.77 ± 0.49*	64.1 ± 6.0*
	5.0	121 ± 11	2.99 ± 0.35*	118 ± 10*
Yukari	1.25	123 ± 12	12.4 ± 1.0	54.1 ± 5.1*
	2.5	123 ± 11	2.35 ± 0.20*	264 ± 21*
	5.0	121 ± 11	ND	> 400
Kuroba	1.25	110 ± 10	12.9 ± 1.0	22.5 ± 2.0*
	2.5	95.1 ± 9.0*	11.0 ± 1.0*	49.0 ± 3.3*
	5.0	70.5 ± 6.5*	7.23 ± 0.65*	79.9 ± 6.7*
Razuberi	1.25	123 ± 12	9.25 ± 0.87*	28.0 ± 2.3*
	2.5	123 ± 11	7.49 ± 0.70*	58.4 ± 5.1*
	5.0	125 ± 11	5.16 ± 0.49*	89.1 ± 8.2*
Benibana	1.25	123 ± 11	7.68 ± 0.72*	68.1 ± 6.1*
	2.5	123 ± 11	4.55 ± 0.40*	143 ± 13*
	5.0	121 ± 11	3.02 ± 0.25*	17.4 ± 1.5*
Burakkuberi	1.25	120 ± 11	10.5 ± 1.0	32.5 ± 2.9*
	2.5	110 ± 10	8.45 ± 0.92*	53.2 ± 5.0*
	5.0	97.5 ± 9.3*	7.91 ± 0.70*	66.1 ± 6.1*
Pepaminto	1.25	83.6 ± 8.0*	3.90 ± 0.25*	126 ± 11*
	2.5	64.3 ± 6.0*	1.82 ± 0.10*	332 ± 25*
	5.0	34.2 ± 3.0*	ND	> 450
Seji	1.25	123 ± 12	13.7 ± 1.3	31.1 ± 2.9*
	2.5	123 ± 11	11.3 ± 1.0*	50.1 ± 4.7*
	5.0	120 ± 11	9.49 ± 0.90*	72.4 ± 6.9*
Orenji	1.25	119 ± 10	10.8 ± 9.5*	34.8 ± 3.0*
	2.5	110 ± 10	9.47 ± 9.0*	49.1 ± 4.0*
	5.0	97.0 ± 9.0*	8.19 ± 0.80*	74.0 ± 6.9*
Kohi	1.25	123 ± 12	5.81 ± 0.49*	60.8 ± 5.9*
	2.5	123 ± 12	1.56 ± 0.10*	304 ± 29*
	5.0	123 ± 12	ND	> 450
Renge	1.25	123 ± 12	12.0 ± 1.1	27.8 ± 2.1*
	2.5	123 ± 12	6.25 ± 0.57*	46.7 ± 4.1*
	5.0	120 ± 11	2.47 ± 0.21*	120 ± 11*
Akashia-C	1.25	123 ± 12	13.6 ± 1.3	24.7 ± 2.0*
	2.5	110 ± 10	10.4 ± 0.95*	37.0 ± 3.2*
	5.0	95.2 ± 9.0*	5.00 ± 0.45*	59.2 ± 5.0*
Satoyama-no-soba	1.25	123 ± 12	13.0 ± 1.2	134 ± 11*
	2.5	122 ± 10	10.3 ± 0.10*	176 ± 15*
	5.0	121 ± 10	9.05 ± 0.85*	260 ± 21*
Satoyama-no-soyogo	1.25	123 ± 12	13.7 ± 1.2	67.6 ± 6.1*
	2.5	111 ± 10	6.63 ± 0.60*	121 ± 10*
	5.0	87.0 ± 8.3*	2.05 ± 0.15*	319 ± 30*
Satoyama-no-renge	1.25	123 ± 12	13.0 ± 1.2	25.4 ± 2.0*
	2.5	120 ± 11	12.9 ± 1.2	36.0 ± 3.0*
	5.0	121 ± 11	12.3 ± 1.1	40.5 ± 3.5*

inhibitory effects on both the rate of amorphous calcium phosphate transformation to hydroxyapatite and the induction time, when used at concentrations of 10–60 μM (Table 5).

Effect of sugars on calcium phosphate precipitation

As shown in Table 6, at a concentration of 2.0% (w/v), glucose had no effect on the rate of amorphous calcium phosphate formation or on the transformation of amorphous calcium phosphate to hydroxyapatite, but increased the induction time to a level 1.3 times higher than that of the control. At a concentration range of 1.0–2.0% (w/v), fructose, sucrose and maltose had no effects on the formation of calcium phosphate precipitates, respectively.

Comparison of honeys with HEBP

The relationship between the induction time and amorphous calcium phosphate transformation to hydroxyapatite was studied using HEBP (10–60 μM) and all of the honeys [2.5% (v/v)] (ratio with respect to the control). As shown in Fig. 2, the curves from both the agents of HEBP and Kanro were essentially the same. This indicated that Kanro was as effective as HEBP. The curves from Rozumari, Razuberi, Pepaminto, Kohi and Satoyama-no-hyakka honeys overlapped with that of Kanro. The curve from Kanro is shown as a representative (curve e in Fig. 2). The curves obtained from Akashia (curve a in Fig. 2), Satoyama-no-soba (curve b in Fig. 2) and Yukari (curve c in Fig. 2) were significantly different from that of HEBP, having a shoulder at a lower concentration. The curves from Manuka, Kuroba and Seji overlapped with that of Akashia. The curves from Satoyama-no-soyogo and Renge overlapped with that of Yukari. The curves from the remaining six types of honey were similar to that of HEBP. The curves from Benibana (curve d) and Petasonkasu (curve f) are shown as representative. Those from Burakkuberi and Orenji overlapped with that of Benibana. Those from Renge and

Table 3. Continued

Honey	Concentration used % (v/v)	Ca^{2+} consumption (p.p.m./min) ACP	Ca^{2+} consumption (p.p.m./min) HAP	Induction time (min)
Satoyama-no-hyakka	1.25	123 ± 11	7.28 ± 0.69*	37.0 ± 3.1*
	2.5	120 ± 11	5.33 ± 0.48*	90.9 ± 8.5*
	5.0	117 ± 10	3.64 ± 0.31*	165 ± 15*

The formation of ACP, the transformation of ACP to HAP and the induction time were measured using the pH drop method. The concentration of calcium and phosphate were 3 mM each. Additives were added to the reaction mixture 5 min before the addition of 3 mM phosphate. The final volume of assay solution, which contains 2 mM Hepes buffer (pH 7.4), was 2 mL.
The reaction mixture was stirred at 37 ± 0.1°C. Values were converted to the rate of consumption of calcium (parts/10^6/min) (14).
ND, not determined.
*Significant difference ($p < 0.05$) when compared with the control (no addition).

Table 4. Effects of propolis on the formation of amorphous calcium phosphate (ACP), the transformation of ACP to hydroxyapatite (HAP) and the induction time

Propolis	Concentration used (μg/mL)	Ca^{2+} consumption (p.p.m./min) ACP	Ca^{2+} consumption (p.p.m./min) HAP	Induction time (min)
None	0	123 ± 12	13.0 ± 1.3	14.8 ± 1.4
Green propolis-1	250	120 ± 11	12.5 ± 1.0	23.8 ± 2.1*
	500	123 ± 12	9.60 ± 0.87*	40.0 ± 3.9*
	1000	123 ± 11	5.66 ± 0.47*	74.5 ± 6.8*
Green propolis-2	250	122 ± 11	7.15 ± 0.61*	33.1 ± 2.1*
	500	124 ± 10	5.46 ± 0.45*	78.7 ± 6.8*
	1000	123 ± 10	4.03 ± 0.37*	158 ± 13*
Green Propolis-3	250	122 ± 10	9.26 ± 0.81*	24.0 ± 2.0*
	500	122 ± 8.0	6.24 ± 0.55*	32.3 ± 2.8*
	1000	123 ± 9.0	3.90 ± 0.27*	141 ± 12*
Brown Propolis	250	121 ± 10	7.02 ± 0.51*	33.4 ± 3.0*
	500	121 ± 11	3.90 ± 0.25*	79.1 ± 6.8*
	1000	121 ± 10	1.56 ± 0.10*	150 ± 13*

The formation of ACP, the transformation of ACP to HAP and the induction time were measured using the pH drop method. The concentration of calcium and phosphate were 3 mM each. Additives were added to the reaction mixture 5 min before the addition of 3 mM phosphate. The final volume of assay solution, which contains 2 mM Hepes buffer (pH 7.4), was 2 mL.
The reaction mixture was stirred at 37 ± 0.1°C. Values were converted to the rate of consumption of calcium (parts/10^6/min) (14).
*Significant difference ($p < 0.05$) when compared with the control (no addition).

Akashia-C overlapped with that of Petasonkasu.

Figure 3 gives a diagrammatic presentation of the results for 20 types of honey and for HEBP. HEBP had almost the maximum effect in terms of the rate of amorphous calcium phosphate transformation to hydroxyapatite (percentage) vs. the induction time plot. The data obtained for honey is presented in Table 3. The range of inhibitory effects of the 20 types of honey varied widely. Ten types of honey (Kanro, Rozumari, Petasonkasu, Yukari, Razuberi, Benibana, Pepaminto, Kohi, Renge and Satoyama-no-hyakka) had inhibitory effects comparable to that of HEBP. Six types of honey (Manuka, Kuroba, Burakkuberi, Seji, Orenji and Akashia-C) resulted in a range of 65–88% of the control in the rate of amorphous calcium phosphate transformation to hydroxyapatite and in an increased induction time of 2.5–4.4 relative to the control. Two types of honey (Akashia and Satoyama-no-renge) showed no inhibition of the rate of amorphous calcium phosphate transformation to hydroxyapatite when used at the lower concentration [2.5% (v/v)], but resulted in an increased induction time of 2.38–2.43-fold. Satoyama-no-soba and Satoyama-no-soyogo increased the induction time by 8.2-fold more than the control value, while exhibiting value of 51–79% of the control in the rate of amorphous calcium phosphate transformation to hydroxyapatite.

Comparison of propolis with HEBP

Similarly, the relationship between the induction time and amorphous calcium phosphate transformation to hydroxyapatite was studied using HEBP (10–60 μM) and four types of propolis (500 μg/mL) (ratio with respect to the control). As shown in Fig. 4, the curves from both HEBP and Green propolis-2 were essentially the same. This indicated that Green propolis-2 was as effective as HEBP. The curve from Green propolis-3 overlapped with that of Green propolis-2. The curve from Green propolis-2 is shown as representative (curve b in Fig. 4). The curve from Brown propolis plotted under that of HEBP (curve c in Fig. 4). The curve from Green propolis-1 (curve a in Fig. 4) was significantly different from that of HEBP, having a shoulder at a lower concentration.

Figure 5 gives a diagrammatic presentation of the results for four types of propolis and HEBP. The data for propolis are presented in Table 4. Three (Green propolis-2, Green propolis-3 and Brown propolis) fell below the curve of HEBP in the range of 42–48% of the control in the rate of amorphous calcium phosphate transformation to hydroxyapatite and in an increased induction time of 2.2–5.3 relative to the control. The remaining type of propolis – Green propolis-1 – was plotted over the curve of HEBP at the 74% of the control in the rate of amorphous calcium phosphate transformation to hydroxyapatite and had an increased induction time of 2.7 fold the control value.

Table 5. Effects of 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) on the formation of amorphous calcium phosphate (ACP), the transformation of ACP to hydroxyapatite (HAP) and the induction time

Concentration used (μM)	Ca^{2+} consumption (p.p.m./min)		Induction time (min)
	ACP	HAP	
None HEBP[a]			
0	123 ± 12	13.0 ± 1.3	14.8 ± 1.4
10	123 ± 12	8.84 ± 0.68*	25.2 ± 2.2*
20	120 ± 10	7.30 ± 0.58*	48.7 ± 4.2*
40	120 ± 10	4.70 ± 0.38*	121 ± 9.6*
60	116 ± 10	2.47 ± 0.15*	221 ± 14*

The formation of ACP, the transformation of ACP to HAP and the induction time were measured using the pH drop method. The concentration of calcium and phosphate were 3 mM each. Additives were added to the reaction mixture 5 min before the addition of 3 mM phosphate. The final volume of assay solution, which contains 2 mM Hepes buffer (pH 7.4), was 2 mL.
The reaction mixture was stirred at 37 ± 0.1°C. Values were converted to the rate of consumption of calcium (parts/10^6/min) (14).
*Significant difference ($p < 0.05$) when compared with the control (no addition).

Table 6. Effects of glucose, fructose, sucrose and maltose on the formation of amorphous calcium phosphate (ACP), the transformation of ACP to hydroxyapatite (HAP) and the induction time

	Concentration used % (w/v)	Ca^{2+} consumption (p.p.m./min)		Induction time (min)
		ACP	HAP	
None	0	123 ± 12	13.0 ± 1.3	14.8 ± 1.4
Glucose	2.0	123 ± 12	11.5 ± 1.0	19.2 ± 1.7*
Fructose	2.0	120 ± 10	13.0 ± 1.3	15.1 ± 1.4
Sucrose	1.0	121 ± 10	13.4 ± 1.3	14.1 ± 1.3
Maltose	1.0	121 ± 12	14.1 ± 1.2	13.7 ± 1.2

The formation of ACP, the transformation of ACP to HAP and the induction time were measured using the pH drop method. The concentration of calcium and phosphate were 3 mM each. Additives were added to the reaction mixture 5 min before the addition of 3 mM phosphate. The final volume of assay solution, which contains 2 mM Hepes buffer (pH 7.4), was 2 mL.
The reaction mixture was stirred at 37 ± 0.1°C. Values were converted to the rate of consumption of calcium (parts/10^6/min) (14).
*Significant difference ($p < 0.05$) when compared with the control (no addition).

Discussion

Using the pH drop method, studies screening natural products have been performed to establish any influence they may have on the *in vivo* oral calcification (14). Because natural products exhibited an inhibitory effect on the precipitation of calcium phosphate, their potential as an anti-calculus agent is suggested, using 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) as the standard (13,18,19). A clinically proven anticalculus agent, such as HEBP, has been shown to chemisorb on the surface of microcrystallites of calcium hydroxyapatite and to prevent further crystal growth, acting as a crystal poison (20). The effectiveness of such agents in inhibiting calcium phosphate precipitation has been evaluated by monitoring either the decrease of the rate of hydroxyapatite crystal growth or the increase in induction time (13,17,21). In our study, eighteen of the honey types investigated (but not Akashia and Satoyama-no-renge) and four types of propolis showed significant inhibitory effects on those reactions.

Ten types of honey (Kanro, Rozumari, Petasonkas, Yukari, Razuberi, Benibana, Pepaminto, Kohi, Renge and Satoyama-no-hyakka) showed

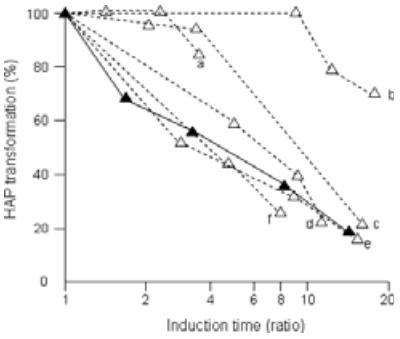

Fig. 2. Relationship between the normalized induction time (ratio to the control) and the rate of hydroxyapatite (HAP) transformation (percentage of control) by 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) and 20 types of honey. The symbols (△) indicate Akashia (a), Satoyama-no-soba (b), Yukari (c), Benibana (d), Kanro (e) and Petasonkas (f), and their concentrations in each curve (from left to right) were 1.25, 2.5 and 5.0% (v/v). The concentrations of HEBP (▲) were (from left to right) 10, 20, 40 and 60 μM. The experimental conditions were the same as those shown in Table 3.

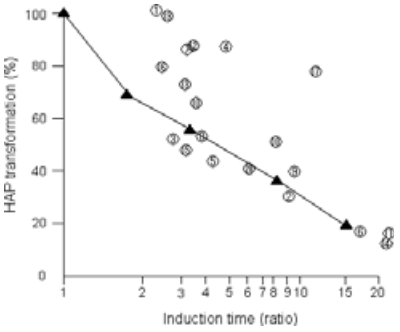

Fig. 3. The ratio of the increase of the induction time and the decrease in hydroxyapatite (HAP) transformation (percentage of the control) of 20 types of honey. The Arabic numeral represents the name of each honey; ① Akasia, ② Kanro, ③ Rozumari, ④ Manuka, ⑤ Petasonkas, ⑥ Yukari, ⑦ Kuroba, ⑧ Razuberi, ⑨ Benibana, ⑩ Burakkuberi, ⑪ Pepaminto, ⑫ Seji, ⑬ Orenji, ⑭ Kohi, ⑮ Renge, ⑯ Akasia-C, ⑰ Satoyama-no-soba, ⑱ Satoyama-no-soyogo, ⑲ Satoyama-no-renge, ⑳ Satoyama-no-hyakka. The concentration was 2.5% (v/v) for the 20 types of honey. For convenience of comparison, the curve for HEBP (▲) is also shown. The concentrations of HEBP were 10, 20, 40 and 60 μM (from left to right).

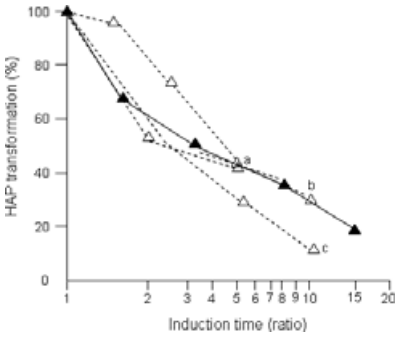

Fig. 4. Relationship between the normalized induction time (ratio to the control) and the rate of hydroxyapatite (HAP) transformation (percentage of control) by 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) and four types of propolis. The symbols (△) indicate Green propolis-1 (a), Green propolis-2 (b) and Brown propolis (c), and their concentrations in each curve (from left to right) were 250, 500 and 1000 μg/mL. The concentrations of HEBP (▲) were (from left to right) 10, 20, 40 and 60 μM. The experimental conditions were the same as those shown in Table 3.

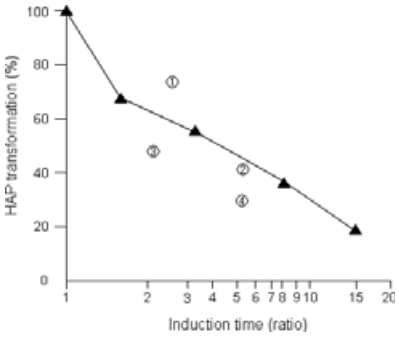

Fig. 5. The ratio of the increase of the induction time and the decrease in hydroxyapatite (HAP) transformation (percentage of the control) of five types of propolis. The Arabic numeral represents the name of each propolis: ① Green propolis-1, ② Green propolis-2, ③ Green propolis-3 and ④ Brown propolis. The concentration was 500 μg/mL for all types of propolis. For convenience of comparison, the curve for 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) (▲) is also shown. The concentrations of HEBP were 10, 20, 40 and 60 μM (from left to right).

almost the same inhibitory effect as HEBP (Table 3 and Fig. 3). In addition, both Satoyama-no-soba and Satoyama-no-soyogo reduced the rate of amorphous calcium phosphate transformation to hydroxyapatite by 21–60% and resulted in 8.5–12-fold increases of the induction time (Table 3 and Fig. 3). Therefore, twelve types of honey may be primary candidates for investigating as anti-calculus agents. However, four of these types of honey (Kanro, Rozumari, Pepaminto and Satoyama-no-soyogo) had an inhibitory effect on the rate of amorphous calcium phosphate formation (Table 3). It is known that the rate of amorphous calcium phosphate formation is decreased by chelating substances such as pyrophosphate, ethyleneglycol-bis-(β-aminoethylether) *N*,*N*′-tetraacetic acid (EGTA) and polyphenols contained in Chinese traditional (Kampo) medicines through the sequestration of calcium from the solutions (13,14). In contrast to HEBP, which inhibits the crystal growth by a threshold mechanism, these 10 types of honey seem to exhibit inhibitory effects by two mechanisms: the inhibition of crystal growth; and the sequestration of calcium from the solutions. Further studies are needed to elucidate these points in greater detail. It should be noted that the chelating property of honeys may present an opportunity to dissolve stones containing calcium salts. However, chelating agents are not appropriate for use in the oral cavity because the composition of calculus is similar to that of cementum and dentin and might therefore erode the teeth. These four types of honey (Kanro, Rozumari, Pepaminto and Satoyama-no-soyogo) should be excluded from the anticalculus agent candidates. Therefore, eight of twelve honeys, namely Petasonkas, Yukari, Razuberi, Benibana, Kohi, Renge, Satoyama-no-hyakka and Satoyama-no-soba, may have the potential for use as anticalculus agents in toothpastes and mouthwashes.

HEBP has been shown to be effective in decreasing the formation of dental calculus in rats (18,22) and in humans (23) and in decreasing the formation of urinary tract stones in human patients at a dose known to affect bone turnover and mineralization (24). As honeys are reported to have no side effects, they are probably safer than synthetic chemicals.

Glucose, fructose and maltose are the main sugars contained in honey. They are generated from invertase-fermented sucrose (25). Furthermore, it has been reported that the content of sugars in honey ranges from 75 to 79% (w/v), of which glucose, fructose, sucrose and maltose represent 31, 38, 1–2 and 7% (w/v), respectively (26). In 5% (w/v) honey, the concentration of glucose, fructose, sucrose and maltose is calculated as 1.6, 1.9, 0.05–0.1, 0.35% (w/v), respectively. At a concentration of 2.0% (w/v), glucose increased the induction time to a level 1.3 times higher than that of the control, whereas at a concentration range of 1.0–2.0% (w/v), fructose, sucrose and maltose had no effects on the formation of calcium phosphate precipitates (Table 6). Therefore, there seems to be no or little effect of the sugars in the honey on the precipitation of calcium phosphate.

Honey color depends on the coloring agents contained in the nectar (xantophile, chlorophyll-like agents, carotene, etc.). That is why the color is connected with a given honey's botanical origin (25). In terms of the color of the honeys, Akashia (Romania) and Satoyama-no-renge (Japan) are pale or light yellow and their inhibition of the calcium phosphate precipitation was weak. Kohi and Pepaminto are brown or dark brown in color and their inhibition was strong. Rozumari, Petasonkas and Razuberi are deep yellow and their inhibition was in-between, or moderate. Therefore, it is thought that there is a correlation between the color of honey and its ability to inhibit calcium phosphate precipitation. Because honey commonly contains flavonoids, which are a plant-coloring material (27), this constituent may be causally related to the inhibitory effect on calcium phosphate precipitation.

It has been reported that sugars can be readily fermented by a wide variety of plaque bacteria to organic acids that can cause caries formation. Among the various sugars, sucrose is unique in that it serves as a specific substrate for bacterial synthesis of extracellular polysaccharides (glucan) (28). As mentioned above, honey contains 31–38% glucose and fructose, 7% maltose and lesser amounts of sucrose (26), whereas

propolis does not contain any sugars (29). Molan (1) has proposed that honey may be cariogenic because of its high content of fermentable sugars; however, with selected honeys that have higher levels of antibacterial activity, the potential for harm to the teeth would be counterbalanced by the inhibition of the cariogenic bacteria.

In terms of the Brazilian propolis, three (Green propolis-2, Green propolis-3 and Brown propolis) fell below the curve of HEBP (Fig. 5). This indicates that these types of propolis may be attractive candidates as anticalculus agents. It has been reported that propolis also contains flavonoids (2,30), the largest and most diverse family of polyphenols (31). As the polyphenols contained in Kampo medicines and their herbal constituents or tea infusions have been found to inhibit the calcium phosphate precipitation (18,19,32), these flavonoids may be an active agent in the honeys and propolis. As the polyphenols have been found to adsorb to hydroxyapatite (18), it is possible that the flavonoids would influence the *in vitro* formation of calcium phosphate precipitates by directly inhibiting crystal growth of hydroxyapatite.

As mentioned above, we have previously studied natural products such as Kampo medicines, herbs and tea infusions, because those have few or no side effects (18,19,32). Two Kampo medicines (Shigyaku-san and Shikunshi-to) have been evaluated as inhibitors of both the *in vitro* formation of calcium phosphate precipitates and the supragingival dental calculus in the rat (18). Therefore, it could be speculated that the inhibitory effect found in an *in vitro* experiment of honeybee products may elicit the same effect *in vivo*.

Both honey and propolis have a long history of being used as folk medicines as well as in medicine to improve the health in the modern era. Our study is the first attempt to apply honeybee products to the area of oral health, such as their potential use as anticalculus agents.

Conclusion

We carried out screening tests of honeys and propolis using a pH drop method. Eight types of honey and three types of propolis were shown to have an efficacy comparable to or even more effective than HEBP in terms of the inhibition of formation of calcium phosphate precipitates. The results of our study will help to elucidate the inhibitory effect of honeybee products on the formation of calculus *in vivo*.

Acknowledgement

This work was supported by a research grant from Yamada Apiculture Center Inc.

References

1. Molan PC. The potential of honey to promote oral wellness. *Gen Dent* 2001;**49:**584–589.
2. Banskota AH, Tezuka Y, Kadota S. Recent progress in pharmacological research of propolis. *Phytother Res* 2001;**15:**561–571.
3. Zumla A, Lulat A. Honey – a remedy of rediscovered. *J R Soc Med* 1989;**82:**384–385.
4. Borchers AT, Mao TK, Keen CL, Schmitz HH, Watanabe H, Gershwin ME. Traditional Asian medicine and oral health. *J Trad Med* 2004;**21:**17–26.
5. Featherstone JD. The continuum of dental caries – evidence for a dynamic disease process. *J Dent Res* 2004; **83** (Spec No C)**:**C39–C42.
6. White DJ. Dental calculus: recent insight into occurrence, formation, prevention, removal and oral health effects of supragingival and subgingival deposits. *Eur J Oral Sci* 1997;**105:**508–522.
7. Mandel ID, Gaffar A. Calculus revisited: a review. *J Clin Periodontol* 1986;**13:**249–257.
8. Fairbrother KJ, Heasman PA. Anticalculus agents. *J Clin Periodontol* 2000;**27:**285–301.
9. Gaffar A, Moreno EC, Muhlemann HR, Niles HP. Effects of EDITEMPA on dental calculus and caries formation in vivo. *Calcif Tissue Int* 1983;**35:**362–365.
10. Wöltgens JH, Qua C, de Blieck-Hogervorst JM. Influence of EHDP and F on Ca and P loss from enamel during the intra-oral cariogenicity test. *J Biol Buccale* 1984;**12:**339–348.
11. White DJ, Gerlach RW. Anticalculus effects of a novel, dual-phase polypyrophosphate dentifrice: chemical basis, mechanism, and clinical response. *J Contemp Dent Pract* 2000;**15:**1–19.
12. Netuveli GA, Sheiham A. A systematic review of the effectiveness of anticalculus dentifrices. *Oral Health Prev Dent* 2004;**2:**49–58.
13. Hidaka S, Abe K, Takeuchi T, Liu SY. Inhibition of the formation of oral calcium phosphate precipitates: beneficial effects of Chinese traditional (Kampo) medicines. *J Periodont Res* 1993;**28:**27–34.
14. Hidaka S, Abe K, Liu SY. A new method for the study of the formation and transformation of calcium phosphate precipitates: effects of several chemical agents and Chinese folk medicines. *Arch Oral Biol* 1991;**36:**49–54.
15. Okamoto Y, Hidaka S. Studies on calcium phosphate precipitation: effects of metal ions used in dental material. *J Biomed Mater Res* 1994;**28:**1403–1410.
16. Hidaka S, Okamoto Y, Abe K. Elutions of metal ions from dental casting alloys and their effect on calcium phosphate precipitation and transformation. *J Biomed Mater Res* 1994;**28:**175–180.
17. Blumenthal NC, Betts SF, Posner AS. Nucleotide stabilization of amorphous calcium phosphate. *Mater Res Bull* 1975;**10:**1055–1060.
18. Hidaka S, Abe K, Liu SY. *In vitro* and *in vivo* evaluations of Chinese traditional (Kampo) medicines as anticalculus agents in the rats. *Arch Oral Biol* 1993;**38:**327–335.
19. Hidaka S, Ouchi K, Yamada Y, Okamoto Y. Influences of tea infusions and glycyrrhiza solution on both the formation of calcium phosphate precipitates and the calcium chelating ability. *J Trad Med* 1998;**15:**147–154.
20. Francis MD. The inhibition of calcium hydroxyapatite crystal growth by polyphosphonates and polyphosphates. *Calc Tiss Res* 1969;**3:**151–162.
21. Root MJ. Inhibition of the amorphous calcium phosphate phase transformation reaction by polyphosphonates and metal ions. *Calc Tiss Res* 1990;**47:**112–116.
22. Briner WW, Francis MD. *In vitro* and *in vivo* evaluation of anti-calculus agents. *Calc Tiss Res* 1973;**11:**10–22.
23. Suomi JD, Horowitz HS, Barbano JP, Spolsky VW, Heifetz SB. A clinical trail of a calculus inhibitory dentifrice. *J Periodontol* 1974;**45:**139–145.
24. Baumann JM, Bisaz S, Fleisch H, Wacker M. Biochemical and clinical effects of ethane-1-hydroxy-1,1′-diphosphonate in calcium nephrolithiasis. *Clin Sci Mol Med* 1978;**54:**509–516.
25. Khismatullina N. *Apitherapy – Guidelines for More Effective use*. Mobile: Perm, 2005:19–34.
26. Kawamura K. Effective components of honeybee products. *Health book Honeybee* 2000;**9:**113–140 (in Japanese).
27. Martos I, Ferreres F, Tomas-Barberan FA. Identification of flavonoid markers for the botanic origin of Eucalyptus honey. *J Agric Food Chem* 2000;**48:**1498–1502.

28. Lingström P, van Houte J, Kashket S. Food starches and dental caries. *Crit Rev Oral Biol Med* 2000;**11:**366–380.
29. Sahinler N, Kaftanoglu O. Naturtal product propolis: chemical composition. *Nat Prod Res* 2005;**19:**183–188.
30. Serra BJ, Soliva TM, Centelles LE. Evaluation of polyphenolic and flavonoid compounds in honeybee-collected pollen produced in Spain. *J Agric Food Chem* 2001;**49:**1848–1853.
31. Bennick A. Interaction of plant polyphenols with salivary proteins. *Crit Rev Oral Biol Med* 2002;**13:**184–196.
32. Hidaka S, Nakajima K, Suekawa M, Liu SY. Saliva and gelatin form precipitates with some traditional Chinese herbs and herbal formulas. *Int J Ori Med* 1995; **20:**169–176.